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(54) Title: METHODS FOR EPITOPE-SPECIFIC AND CYTOKINE/ANTICYTOKINE COMBINATION IMMUNOTHERAPIES FOR MODULATION OF PATHOGENIC IMMUNE RESPONSES IN IMMUNE MEDIATED DISEASES

(57) Abstract: The current invention provides for methods of immunotherapy using a combination of epitope-specific and cytokine or anticytokine immunotherapy. The method provides for modulation of pathogenic immune responses and includes the identification of molecules comprising specific epitopes involved in a particular disease state of interest, administration of the epitope-specific molecule in conjunction with the cytokine or anticytokine, and downstream modification of the administration of the cytokine/anti-cytokine relative to the administration of the epitope-specific molecule.

**METHODS FOR EPITOPE-SPECIFIC AND CYTOKINE/ANTICYTOKINE  
COMBINATION IMMUNOTHERAPIES FOR MODULATION OF  
PATHOGENIC IMMUNE RESPONSES IN IMMUNE MEDIATED DISEASES**

**GOVERNMENT INTERESTS**

[0001] This invention was made with Government support under Grant No. AR44850, awarded by the National Institutes of Health. Accordingly, the U.S. Government may have certain rights in this invention.

**FIELD OF THE INVENTION**

[0002] This invention relates generally to immunotherapy methodologies in immune mediated disease states and, more specifically, to use of combination therapies to stimulate immune cells *in vivo* to cause modulation of pathogenic responses.

**BACKGROUND OF THE INVENTION**

[0003] It has long been recognized that immune inflammation comes about by a combination of antigen-specific and non-specific immune responses to a given stimulus. Antigen-specific responses comprise in part, T and B cell recognition of self and/or exogenous proteins or peptides, or even mixtures of lipids, carbohydrates and peptides, either soluble, complexed to antibodies, or processed into immunogenic peptides (i.e. epitopes) by antigen presenting cells. Recognition of an antigen leads to a response by the specific effector B or T cells and consists of a combination of cell proliferation, soluble mediator (i.e. cytokines) and/or effector (i.e. antibodies) secretion, and the expression of transmembrane proteins which govern the intensity and duration of the response through direct cell to cell contact or by soluble mediators. Under physiologic conditions, such antigen-specific responses are usually self-limited and appear to control non-specific immune mechanisms. The recognition of an antigen can also lead directly to interference with downstream events, through anergy or clonal deletion of specific cells.

[0004] Many treatment regimens have been developed for the treatment of rheumatoid arthritis and other autoimmune diseases with which immune inflammation is

a complication. For example, "biologic therapies" have brought about numerous advances in the therapeutic approach to these diseases. Such therapies often include use of genetically engineered proteins. For example, monoclonal antibodies and receptor-immunoglobulin fusion proteins designed to modulate specific underlying autoimmune processes can be used and often allow for avoidance of certain problematic effects such as generalized immunosuppression. Such therapies also include use of compounds that interfere with the "trimolecular complex" that comprises the major histocompatibility complex II-Antigen-T cell receptor interactions.

[0005] Other treatment regimens are designed to block secondary signals for T cell activation and T cell interaction by using antigen-presenting cells, and cytokine agonists as well as antagonists.

[0006] Still other treatment therapies include regimens designed to affect tumor necrosis factor alpha (TNF $\alpha$ ), which is a pivotal cytokine in the inflammatory process. For example, in one such regimen, anti-TNF reagents are used to interfere with the TNF pathway to provide short-term clinical efficacy and tolerability. Such anti-TNF reagents include infliximab which is a chimeric monoclonal anti-TNF $\alpha$ , soluble TNF $\alpha$  receptors, etanercept, and talidomide. Such therapeutic compounds also affect cytokines, for example, interleukin-1 (IL-1), wherein production of IL-1 *in vivo* is blocked. The blocking of IL-1 production may have beneficial effects, although the nature of such effects is still to be determined.

[0007] The use of etanercept, a fusion protein consisting of the extracellular ligand binding domain of the 75kD receptor for TNF $\alpha$  and the constant portion of human IgG1, alone or in combination with methotrexate for treating patients having active rheumatoid arthritis (defined by the American College of Rheumatology (ACR) as functional class I to III) and who had previously failed to respond to treatment with greater than or equal to one disease-modifying antirheumatic drug (DMARD), produced improvements in all core ACR measures of disease activity.

[0008] Although the various above stated therapy regimens have shown a degree of success in treating autoimmune diseases, problems associated with long-term

administration of such biological agents are not yet known. Both IL-1 and TNF $\alpha$  play important roles in normal host defenses and complications from blocking their production or effects may develop in patients. Ultimately, there is a need for careful evaluation of such therapeutics in long-term studies. There is also concern that such therapies may increase the rate of serious infections and allow for a reduced degree of control over neoplastic cells, especially in patients with severe disease. The development of antigen responses to injected monoclonal antibodies and poor pharmacokinetics of low molecular-weight inhibitors are additional problems.

[0009] Other advances in treatment of autoimmune diseases include the limited use of combination-oriented therapies. For example, a treatment regimen for experimental autoimmune encephalomyelitis (EAE) has been tested using antigen-specific immunotherapy combined with cytokine therapy. In this example, local gene delivery of the interleukin-4 (IL-4) gene was administered with a tolerizing DNA vaccination; the DNA of the vaccine encoding a self-peptide proteolipid protein. This therapy demonstrated that co-delivery of two different DNAs provides protective immunity against EAE and reverse established EAE by the expression of IL-4 from the delivered naked DNA, which is secreted and acts locally on autoreactive T cells, causing the cells to shift their cytokine profile to Th 2. This treatment strategy therefore combined the antigen-specific effects of DNA vaccination and the beneficial effects of local gene delivery. However, the methodology of the treatment provided no directed control over the desired immune modulation. The ultimate efficacy of such a regimen is unknown because there is no demonstration of the capability to control the modulation of immune response.

[0010] Thus, a need exists for additional combination therapies that can more directly provide immune modulation in the treatment of immune diseases without compounding side effects. The present invention satisfies this need and provides additional advantages through a broad-based approach to immunotherapy for immune mediated diseases that allows for the controlled modulation of pathogenic immune response using a combination of epitope-specific and cytokine or anticytokine immunotherapy.

SUMMARY OF THE INVENTION

[0011] In a first embodiment, the methods of the invention provide for the combination of epitope-specific and cytokine/anticytokine therapy to achieve effective control of immune mediated disease processes. In a particularly preferred embodiment, the methods of the invention provide for a continuing regulation of immunity and control of disease manifestations through up or down regulation of innate and acquired immunity. Examples of diseases for which such methods are contemplated to be effective include, but are not limited to, rheumatoid arthritis, diabetes type I, multiple sclerosis, and inflammatory bowel disease.

[0012] In another embodiment, the methods of the invention provide for combining mechanisms for identifying and modulating epitope-specific immune responses with strategies to up or down regulate cytokine mediated responses. In another embodiment the methods of the invention lead to specific and controlled modulation of pathogenic immune responses, and eventually to control of disease in a physiologic, non-toxic and cost effective manner. Embodiments of the invention contemplate regulation of cytokine mediated responses related to inflammatory and/or tolerogenic responses associated with immunomodulatory cytokines including, but not limited to, TNF $\alpha$ , TNF $\beta$ , IFN $\alpha$ , IFN $\beta$  or IFN $\gamma$ , IL-1, IL-4, IL-6, IL-10, IL-15 and IL-23.

[0013] In a further embodiment, the methods of the invention provide for modulating a pathogenic immune response such that the response reverts to the point in time of immune response wherein antigen-specific events are dominant and potentially relevant to disease outcome. By "dominance" of antigenic events is meant that the influence of such events is relevant enough to trigger, perpetuate or affect in any way disease pathogenesis and outcome. By "potentially relevant" is meant that the influence is theorized but not proven, and by "relevant" is meant that there is evidence to support a role in the pathogenesis. In a related embodiment, the combination of anticytokine therapy with epitope-specific therapy enables intervention of antigen-specific events to permanently and specifically induce desired antigen-specific modulation. In a

particularly preferred embodiment, such modulation leads to clinically detectable amelioration of disease.

[0014] In a further related embodiment, the methods of the invention provide amelioration of disease in an antigen-specific fashion, while reducing the need for ongoing toxic, non-specific, uncontrolled, and expensive cytokine/anticytokine therapy.

[0015] In a further embodiment, the invention provides a method of modulating an immune response in a subject having an immune response-related disorder. The method includes a) administering a therapeutically effective amount of a an antigen-specific epitope, wherein administration provides epitope-specific T cell immune modulation; and b) administering a therapeutically effective amount of a cytokine or a regulatory effective amount of an agent that affects cytokine activity or expression, wherein administration of a) and b) modulates the immune response in the subject.

[0016] In another embodiment, the invention includes a method of modulating immune processes and pathogenesis of immune mediated diseases. The method includes a) identifying at least one suitable epitope; b) identifying at least one cell type associated with the immune mediated disease; and c) administering a combination of antigen-specific and cytokine or anticytokine therapy wherein the administering induces an immune response to the immune mediated disease.

[0017] In yet another embodiment, the invention provides a method of treating a subject having an immune response-related disorder. The method includes a) administering a therapeutically effective amount of a an antigen-specific epitope, wherein administration provides epitope-specific T cell immune modulation; and b) administering a therapeutically effective amount of a cytokine or a regulatory effective amount of an agent that affects cytokine activity or expression, wherein administration of a) and b) modulates the immune response in the subject, thereby treating the subject.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0018] Figure 1 is a graph showing the effects on mean arthritis scores calculated for rats divided into four groups following induction of Adjuvant Arthritis.

The four treatment regimens were: (1) Etanercept alone injected 3 times; (2) Etanercept alone injected 1 time; (3) Etanercept injected 1 time with mucosal administration of mycobacterial heat shock protein 180-188; and (4) animals receiving no treatment.

[0019] Figure 2 is a set of graphs showing the stimulation of mandibular lymph node cells for each of the four test groups shown in Fig. 1. The bars labeled 180-188, Mt, and OVA represent that the culture conditions used these as antigens in the media for accessing the stimulation index.

[0020] Figure 3 is a set of bar graphs showing intracellular cytokine production in inguinal lymph nodes (ILN) by FACS analysis. In the first graph is the percentage of CD4+ cells found expressing IL-10. The identification of CD4+ cells which express CTLA-4 and produce IL-10 is a requisite of T regulatory cells.

[0021] Figure 4 is a bar graph showing the gene transcription of IL-10 by Taqman technique which is a real time PCR technique.

[0022] Figure 5 is a bar graph showing gene transcription of TGF $\beta$  by Taqman technique for mandibular lymphocytes (MLN) and (ILN).

[0023] Figure 6 is a bar graph showing gene transcription of GATA-3 by the Taqman technique.

[0024] Figure 7 is a bar graph showing gene transcription of Tbet by Taqman technique.

#### DETAILED DESCRIPTION OF THE INVENTION

[0025] The methods of the invention are directed to methods of immunotherapy that stimulate immune cells to cause modulation of pathogenic responses with limited long term effects in a non-toxic and cost effective manner. By use of the methods of the invention, pathogenic immune responses may be controlled and/or modulated. Accordingly, the autoimmune diseases from which the pathogenic response comes may also be controlled or ameliorated.

[0026] Immunotherapy is a process by which the cells of the immune system are used or stimulated in an attempt to fight disease, infection or growth. Such therapy may be passive or active. Passive immunotherapy involves direct administration of the substances (e.g., antibodies) that fight the disease. Active immunotherapy, contrarily, involves administration of the substances that stimulate the cells of the immune system to produce their own natural substances that fight the disease.

[0027] In one embodiment, the invention comprises methods wherein an immune response may be modulated by administration of a therapeutically effective amount of an antigen-specific epitope and a therapeutically effective amount of a compound that regulates the pathophysiologic role of a cytokine.

[0028] The administration of the compounds in the methods of the invention may be performed by any of the methods known to those of skill in the art. Such methods include, but are not limited to, direct administration by mucosal, intravenous (iv), subcutaneous (sc), or intramuscular (im) administration.

[0029] The invention methods include identification of proteins from T cell epitopes, where the proteins are targets of T cell immune responses. By "T cell epitope(s)" is meant a contiguous amino acid sequence recognized by a T cell in the context of the restricting MHC molecule. In one embodiment, the invention methods comprise a combination of epitope composition prediction within a protein by computer programming and actual epitope mapping methods. These computer programs and mapping techniques are well understood by those of skill in the art. In a related embodiment, epitope-specific T cells are characterized phenotypically and functionally. By "phenotypically" is meant a phenotypic characterization that identifies membrane proteins. By "functionally" is meant a characterization that identifies cell responses related to antigens e.g., cell proliferation and production of inter- and intracellular mediators. Such characteristics are then correlated with clinical variables, including disease activity and gravity. Epitopes that are relevant to a given pathogenic pathway are thereby identified.

[0030] Epitopes found to be relevant to a given pathogenic pathway are also therefore related to either up- or down-regulation of inflammation in any particular disease state for which epitopes are sought. Down-regulation is desirable in those morbid conditions, such as autoimmunity, in which control of self reactivity may lead to clinical improvement. Up-regulation is desirable in those diseases in which generation of inflammation may lead to improved defenses. Examples of such diseases include, but are not limited to, infection caused by foreign biological and chemical agents and cancer.

[0031] In one embodiment of the invention, epitopes, or epitope-specific T cells, are identified and characterized by, but not limited to:

- i) Computerized analysis of putative epitopes on immunologically relevant proteins (e.g., see U.S. Patent No. 6,037,135.);
- ii) Evaluation of antigenicity in animal models *in vivo* and *in vitro* and in human diseases *in vitro* or *ex vivo* by means known to those skilled in the art;
- iii) Identification and isolation of epitope-specific T cells from patients' samples by fluororochrome tagged artificial antigen presenting cells (such as described in PCT/US99/24666); and
- iv) Characterization of isolated epitope-specific T cells by means known to those skilled in the art.

[0032] Specific examples of antigen-specific epitopes further include epitopes in any of the following peptides in Table 1. These peptides include, but are not limited to, derivatives or mutants/analogs of heat shock 60 (HSP60) proteins and dnaJ proteins of human and prokaryotic, (i.e., mycobacteria or *E. coli*) origin.

**Table 1**

Origin	Protein	Position	Sequence
<i>E. coli</i>	dnaJp2		VLTDQSQKRRAYDQYQG (SEQ ID NO: 1)
<i>E. coli</i>	dnaJ 4		QDYYEILGVSKTAAEE (SEQ ID NO: 2)
<i>E. coli</i>	dnaJ 22		RKAYKRLAMKYHPDR (SEQ ID NO: 3)
<i>E. coli</i>	dnaJ 61		QKRAA YDQYGHAAFEQ (SEQ ID NO: 4)
<i>E. coli</i>	dnaJ 174		QGFFAVQQTCPHCQG (SEQ ID NO: 5)
<i>E. coli</i>	dnaJ 209		SKTLSVKKPQAVDTG (SEQ ID NO: 6)
<i>E. coli</i>	dnaJ 242		GDLYVQVQVKQHPIF (SEQ ID NO: 7)
<i>E. coli</i>	dnaJ 264		YCEVPINPAMAAALGG (SEQ ID NO: 8)
<i>E. coli</i>	dnaJ 268		PINFAMAAALGGEIEV (SEQ ID NO: 9)
<i>E. coli</i>	DnaJp1		QKRAA YDQYGHAAFE (SEQ ID NO: 10)
<i>E. coli</i> mutant	dnaJpV		DERAAYDQYGHAAFE (SEQ ID NO: 11)
<i>E. coli</i>	HSP60	212-226	AVELESPFILLADKK (SEQ ID NO: 12)
<i>E. coli</i>	HSP60	218-232	PFILLADKKISNIKE (SEQ ID NO: 13)
<i>E. coli</i>	HSP60	256-270	GEALATLVVNTMRGBI (SEQ ID NO: 14)
<i>E. coli</i>	HSP60	510-524	VAGLMITTECMVTDL (SEQ ID NO: 15)
human	S1 HLA		QKRAAVDTYCRHNYG (SEQ ID NO: 16)
human	S2 HLA		KDLLEQKRAAVDTC (SEQ ID NO: 17)
human	2 (HSJ1)		ASYYEILDVPRASA (SEQ ID NO: 18)
human	3 (HDJ1)		KDYYQTLGLARGASD (SEQ ID NO: 19)
human	5 (HDJ2)		TTYYDVLGVKPNATQ (SEQ ID NO: 20)
human	20 (HSJ1)		KKAYRKRALQWHPDK (SEQ ID NO: 21)
human	21 (HDJ1)		KRAYRRQALRYHPDK (SEQ ID NO: 22)
human	23 (HDJ2)		KKAYRKRALKYHPDK (SEQ ID NO: 23)
human	164 (HSJ1)		FRSVSTSTTFVQGRR (SEQ ID NO: 24)
human	167 (HDJ2)		PGMVQQIQSVCMECQ (SEQ ID NO: 25)
human	176 (HSJ1)		GRRITTRIMENGQE (SEQ ID NO: 26)
human	50 (HDJ2)		QAYEVLSDAKKRELYD (SEQ ID NO: 27)
human	51 (HSJ1)		EAYEVLSDKHKREIYD (SEQ ID NO: 28)
human	134 (HSJ1)		SGPFTFTSSSFPGHS (SEQ ID NO: 29)
human	197 (HSJ1)		DGQLKSVTINGVPDD (SEQ ID NO: 30)
human	254 (HSJ1)		DLQLAMAYSLSEMEA (SEQ ID NO: 31)
human	256 (HDJ2)		EDLFMCMIDIQLVEAL (SEQ ID NO: 32)
human	270 (HDJ2)		LCGFQKPISTLDNRT (SEQ ID NO: 33)
human	283 (HDJ2)		RTIVITSHPGQIVKH (SEQ ID NO: 34)
human	318 (HDJ2)		GRLIIEFKVNFPENG (SEQ ID NO: 35)
human	HSP60	105-127	TNEEAGDGTITATVLARSIAKEG (SEQ II NO: 36)
human	HSP60	195-226	RKGVITVKDGKTLNDELEIIEGMKFDRG ISP (SEQ ID NO: 37)
human	HSP60	234-266	GQKCEFDQDAYVLLSEKKISSIQSIVPALEI ANA (SEQ ID NO: 38)
human	HSP60	236-250	KCEFQDAYVLLSEKK (SEQ ID NO: 39)

<u>Origin</u>	<u>Protein</u>	<u>Position</u>	<u>Sequence</u>
human	HSP60	242-256	AYVLLSEKKKISSIQS (SEQ ID NO: 40)
human	HSP60	269-307	KPLVIIAEDVDGEALSTLVNLKVGQLQVAVKAPGFD (SEQ ID NO: 41)
human	HSP60	280-294	GEALSTVLVNLKVG (SEQ ID NO: 42)
human	HSP60	410-445	SDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALL (SEQ ID NO: 43)
human	HSP60	469-502	KRTLKIPAMTIAKNAGVEGSLIVEKIMQS SSE (SEQ ID NO: 44)
human	HSP60	523-656	KVVRTALLDAAGVASLLTAEVVVTEIP (SEQ ID NO: 45)
human	HSP60	535-549	VASLLTTAEVVVTEI (SEQ ID NO: 46)
mycobacterium	HSP60	80-102	TDDVAGDGTTTATVLAQALVREG (SEQ ID NO: 47)
mycobacterium	HSP60	169-200	NEGVITVEESNTFGLQLELTEGMRFDKG <sup>1</sup> ISG (SEQ ID NO: 48)
mycobacterium	HSP60	180-188	TFGLQLELT (SEQ ID NO: 49)
mycobacterium	HSP60	208-240	RQEAVLEDPYIILLVSSKVSTVKDPLLKEVIG (SEQ ID NO: 50)
mycobacterium	HSP60	210-224	EALEDPYIILLVSSK (SEQ ID NO: 51)
mycobacterium	HSP60	216-230	PYILLVSSKVSTVKD (SEQ ID NO: 52)
mycobacterium	HSP60	243-281	KPLLIIAEDVGEALSTLVNKIRGTFKS <sup>1</sup> AVKAPGFD (SEQ ID NO: 53)
mycobacterium	HSP60	254-268	GEALSTLVVNKIRGT (SEQ ID NO: 54)
mycobacterium	HSP60	383-418	TEVELKERKHRIEDAVRNAAVVEEGIVAGGGVTL (SEQ ID NO: 55)
mycobacterium	HSP60	441-478	KVALEAPLKQIAFNSGLEPGVVAEKVRNLPAG (SEQ ID NO: 56)
mycobacterium	HSP60	494-527	KVTRTSALQNAASIAGLFLTTEAVVADKPK. KEKA (SEQ ID NO: 57)
mycobacterium	HSP60	503-517	IAGLFLTTEAVVADK (SEQ ID NO: 58)
mycobacterium	HSP60	507-521	IAGLFLTTEAVVADK (SEQ ID NO: 59)

**[0033]** In still another embodiment, immunotherapy is carried out in at least two distinct phases comprising "induction" and "consolidation" therapy periods.

**[0034]** By "induction period" is meant a period of time used for down-regulation of non-specific inflammation to allow for generation of regulatory epitope-specific T cell responses. In this time period, a subject patient is treated with a combined administration of compounds that up- or down-regulate cytokines important in a particular disease state

of interest with antigen-specific immune modulation. The objective of this treatment period is to create an appropriate environment for antigen-specific T cell regulation.

[0035] The immunological environment to be generated depends on whether there is a need for up- or down-regulation. For example, for those cases requiring down-regulation of inflammation (e.g., autoimmunity), blocking, inhibiting or destruction of one or more pro-inflammatory or activating cytokines is carried out. Conversely, when up-regulation of antigen-specific T cell responses is desired immunostimulatory cytokines are employed to boost defensive immune responses. Up-regulation may be desired, for example, in infectious disease treatments, vaccinations and cancer regimens. Immunomodulatory cytokines that can be used in the above stated up or down regulation, depending upon the desired outcome include, but are not limited to IFN $\alpha$ , IFN $\beta$ , IFN $\gamma$ , TNF $\alpha$ , TNF $\beta$ , TGF $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12, IL-15 and IL-23.

[0036] As used herein the term "cytokine" encompasses chemokines, interleukins, lymphokines, monokines, colony stimulating factors, and receptor associated proteins, and functional fragments thereof. As used herein, the term "functional fragment" refers to a polypeptide which possesses biological function or activity that is identified through a defined functional assay.

[0037] The cytokines, colony stimulating factors (CSF), and interleukins (IL) useful as a proteinaceous ligand moiety include endothelial monocyte activating polypeptide II (EMAP-II), granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, and IL-13, and the like, which bind, respectively, to the EMAP-II, GM-CSF, G-CSF, M-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, IL-13 families of cytokine receptors, and the like, and which is associated with a particular biologic, morphologic, or phenotypic alteration in a cell or cell mechanism.

[0038] Soluble receptors of cytokines may also be utilized in the methods of the invention by acting as anti-cytokines in that they can be administered to compete with the natural target of the cytokines. Examples of soluble receptors applicable to the current invention include, but are not limited to, soluble receptors specific for IL-1 and

TNF $\alpha$ . The production of such soluble receptors and their respective use are well known to those of skill in the art.

[0039] Cytokine antagonists can take several forms. They may be monoclonal antibodies. They may be a monoclonal antibody fragment. They may take the form of a soluble receptor to that cytokine. Soluble receptors freely circulate in the body. When they encounter their target cytokine they bind to it, effectively inactivating the cytokine, since the cytokine is then no longer able to bind with its biologic target in the body. Thus, a cytokine antagonist of the invention also includes soluble receptors or fragments thereof. An even more potent antagonist consists of two soluble receptors fused together to a specific portion of an immunoglobulin molecule (Fc fragment). This produces a dimer composed of two soluble receptors which have a high affinity for the target, and a prolonged half-life. This new molecule is called a fusion protein. An example of this new type of molecule, called a fusion protein, is etanercept (Enbrel $\circledR$ ).

[0040] TNF, a naturally occurring cytokine, plays a key role in the inflammatory response, in the immune response, and in the response to infection. TNF is formed by the cleavage of a precursor transmembrane protein, forming soluble molecules which aggregate in vivo to form trimolecular complexes. These complexes then bind to receptors found on a variety of cells. Binding produces an array of effects, including release of other immunomodulatory cytokines, including IL-6, IL-8, and IL-1; release of matrix metalloproteinases; and up regulation of the expression of endothelial adhesion molecules, further amplifying the inflammatory and immune cascade by attracting leukocytes into extravascular tissues.

[0041] Interleukin-1 (IL-1) is a cytokine which has been implicated in the inflammatory response occurring in the brain, spinal cord, retina, muscle, and elsewhere in the body. There are two naturally occurring inhibitors of IL-1 in the body: IL-1 receptor antagonist (IL-1 RA) and IL-1 receptor type II (IL-1 R type II). Additional inhibitors of IL-1 for the purpose of this patent are soluble IL-1 receptors: fusion proteins consisting of two IL-1 receptors attached to the Fc portion of a human IgG molecule (IL-1 R-FP); and monoclonal antibodies with a high affinity for IL-1. IL-6 and IL-8 are both also immunomodulatory cytokines.

[0042] Agents useful for administration to a subject for regulating cytokine activity or expression or a cytokine receptor also include antibodies or fragments thereof, a peptide, a peptidomimetic, a polynucleotide, a small organic molecule, or any other agent, that can act directly or indirectly as an agonist of cytokine signal transduction or as an antagonist of cytokine signal transduction. In another embodiment, the agent can specifically interact with a cytokine or cytokine receptor expressed by a cell. For example, a peptide agent is exemplified by an anti-cytokine or cytokine receptor antibody or by an anti-idiotypic antibody of an anti-cytokine or cytokine antibody. Such a peptide agent provides the additional advantage that it can be selected not only for its ability to interact specifically with a cytokine or cytokine receptor, thereby competing with cytokine or cytokine for the receptor, but can be further selected to have an ability to not activate cytokine signal transduction for example.

[0043] As used herein, the term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies. An antibody useful in a method of the invention, or an antigen binding fragment thereof, is characterized, for example, by having specific binding activity for an epitope. In addition, as discussed above, an antibody of the invention can be an antibody that specifically binds a peptide portion of a polypeptide.

[0044] The term "binds specifically" or "specific binding activity," when used in reference to an antibody means that an interaction of the antibody and a particular epitope has a dissociation constant of at least about  $1 \times 10^{-6}$ , generally at least about  $1 \times 10^{-7}$ , usually at least about  $1 \times 10^{-8}$ , and particularly at least about  $1 \times 10^{-9}$  or  $1 \times 10^{-10}$  or less. As such, Fab, F(ab')<sub>2</sub>, Fd and Fv fragments of an antibody that retain specific binding activity for an epitope, are included within the definition of an antibody. For purposes of the present invention, an antibody may specifically react with an epitope of a cytokine or cytokine receptor, for example, is considered to not substantially cross react with another cytokine or cytokine receptor if the antibody has at least a two-fold greater binding affinity, generally at least a five-fold greater binding affinity, and particularly at least a ten-fold greater binding affinity for the cytokine or cytokine receptor as compared to the related cytokine or cytokine receptor.

[0045] The term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains (see Huse et al., *Science* 246:1275-1281 (1989).). These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, *Immunol. Today* 14:243-246, 1993; Ward et al., *Nature* 341:544-546, 1989; Harlow and Lane, *Antibodies: A laboratory manual* (Cold Spring Harbor Laboratory Press, 1988); Hilyard et al., *Protein Engineering: A practical approach* (IRL Press 1992); Borrabeck, *Antibody Engineering*, 2d ed. (Oxford University Press 1995).).

[0046] If desired, a kit incorporating an antibody or other agent useful in a method of the invention can be prepared. Such a kit can contain, in addition to the agent, a pharmaceutical composition in which the agent can be reconstituted for administration to a subject. The kit also can contain, for example, reagents for detecting the antibody, or for detecting specific binding of the antibody to a cytokine or cytokine receptor. Such detectable reagents useful for labeling or otherwise identifying the antibody are described herein and known in the art.

[0047] Methods for raising polyclonal antibodies, for example, in a rabbit, goat, mouse or other mammal, are well known in the art (see, for example, Green et al., "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed., Humana Press 1992), pages 1-5; Coligan et al., "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters," in *Curr. Protocols Immunol.* (1992), section 2.4.1.). In addition, monoclonal antibodies can be obtained using methods that are well known and routine in the art (Harlow and Lane, *supra*, 1988). For example, spleen cells from a mouse immunized with a cytokine or cytokine receptor, or an epitopic fragment thereof, can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using labeled antigen to

identify clones that secrete monoclonal antibodies having the appropriate specificity, and hybridomas expressing antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of the antibodies. The antibodies can be further screened for the inability to bind specifically with the cytokine or cytokine receptor. Such antibodies are useful, for example, for preparing standardized kits for clinical use. A recombinant phage that expresses, for example, a single chain anti-cytokine or cytokine receptor antibody also provides an antibody that can be used for preparing standardized kits.

[0048] Methods of preparing monoclonal antibodies well known (see, for example, Kohler and Milstein, *Nature* 256:495, 1975; see, also, Coligan et al., *supra*, 1992, see sections 2.5.1-2.6.7; Harlow and Lane, *supra*, 1988). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

[0049] Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well established techniques, including, for example, affinity chromatography with Protein-A SEPHAROSE, size exclusion chromatography, and ion exchange chromatography (Coligan et al., *supra*, 1992, see sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; see, also, Barnes et al., "Purification of Immunoglobulin G (IgG)," in *Meth. :Molec. Biol.* 10:79-104 (Humana Press 1992)). Methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies is well known to those skilled in the art. Multiplication *in vitro* can be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer

reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* can be carried out by injecting cell clones into mammals histocompatible with the parent cells, for example, syngeneic mice, to cause growth of antibody producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

[0050] Therapeutic applications for antibodies disclosed herein are also part of the present invention. For example, antibodies of the present invention can also be derived from subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg et al., International Patent Publication WO 91/11465 (1991); and Losman et al., *Int. J. Cancer* 46:310, 1990.

[0051] A therapeutically useful anti-cytokine or cytokine receptor antibody also can be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are known (see, for example, Orlandi et al., *Proc. Natl. Acad. Sci., USA* 86:3833, 1989.). Techniques for producing humanized monoclonal antibodies also are known (see, for example, Jones et al., *Nature* 321:522, 1986; Riechmann et al., *Nature* 332:323, 1988; Verhoeven et al., *Science* 239:1534, 1988; Carter et al., *Proc. Natl. Acad. Sci., USA* 89:4285, 1992; Sandhu, *Crit. Rev. Biotechnol.* 12:437, 1992; and Singer et al., *J. Immunol.* 150:2844, 1993.).

[0052] Antibodies of the invention also can be derived from human antibody fragments isolated from a combinatorial immunoglobulin library (see, for example, Barbas et al., *METHODS: A Companion to Methods in Immunology* 2:119, 1991; Winter et al., *Ann. Rev. Immunol.* 12:433, 1994.). Cloning and expression vectors that are useful

for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, CA).

[0053] An antibody of the invention also can be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green et al., *Nature Genet.* 7:13, 1994; Lonberg et al., *Nature* 368:856, 1994; and Taylor et al., *Int. Immunol.* 6:579, 1994.

[0054] Antibody fragments of the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')<sub>2</sub>. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulphydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see, for example, Goldenberg, U.S. Patent No. 4,036,945 and U.S. Patent No. 4,331,647; and references contained therein; Nisonhoff et al., *Arch. Biochem. Biophys.* 89:230, 1960; Porter, *Biochem. J.* 73:119, 1959; Edelman et al., *Meth. Enzymol.*, 1:422 (Academic Press 1967); see, also, Coligan et al., *supra*, 1992, see sections 2.8.1-2.8.10 and 2.10.1-2.10.4).

[0055] Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light/heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques can also be used, provided the fragments specifically bind to the antigen that is recognized by the intact antibody. For example,

Fv fragments comprise an association of V<sub>H</sub> and V<sub>L</sub> chains. This association can be noncovalent (Inbar et al., *Proc. Natl. Acad. Sci., USA* 69:2659, 1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde ( Sandhu, *supra*, 1992). Preferably, the Fv fragments comprise V<sub>H</sub> and V<sub>L</sub> chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V<sub>H</sub> and V<sub>L</sub> domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow et al., *Methods: A Companion to Methods in Enzymology* 2:97, 1991; Bird et al., *Science* 242:423-426, 1988; Ladner et al., U.S. patent No. 4,946,778; Pack et al., *Bio/Technology* 11:1271-1277, 1993; see, also Sandhu, *supra*, 1992.

[0056] Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (see, for example, Larrick et al., *Methods: A Companion to Methods in Enzymology* 2:106, 1991.).

[0057] Antibodies (immunoglobulins) are proteins produced by one class of lymphocytes (B cells) in response to specific exogenous foreign molecules (antigens). Monoclonal antibodies (mAB), identical immunoglobulin copies which recognize a single antigen, are derived from clones (identical copies) of a single B cell. This technology enables large quantities of an immunoglobulin with a specific target to be mass produced.

[0058] Monoclonal antibodies with a high affinity for a specific cytokine will tend to reduce the biologic activity of that cytokine. Substances which reduce the biologic effect of a cytokine can be described in any of the following ways: as a cytokine

blocker; as a cytokine inhibitor; or as a cytokine antagonist. In this patent application, the terms blocker, inhibitor, and antagonist are used interchangeably with respect to cytokines.

[0059] Advances in biotechnology have resulted in improved molecules as compared to simply using monoclonal antibodies. One such molecule is an antibody fragment. By removing part of the antibody structure, the function of this molecule is changed so that it acts differently in the human body. Another new type of molecule, distinct from monoclonal antibodies and soluble receptors, is a fusion protein. Such molecules have a distinct function which acts differently in the human body than a simple soluble receptor or receptors.

[0060] An agent useful in a method of the invention also can be a polynucleotide. Generally, but not necessarily, the polynucleotide is introduced into the cell, where it effects its function either directly, or following transcription or translation or both. For example, the polynucleotide agent can encode a peptide, which is expressed in the cell and modulates a cytokine or cytokine receptor activity. Such an expressed peptide can be, for example, a mutant cytokine or cytokine receptor such as a soluble cytokine or cytokine receptor extracellular domain; a cytokine or cytokine receptor extracellular domain operatively associated with a membrane anchoring domain; or a mutant cytokine or cytokine receptor lacking activity.

[0061] A polynucleotide of the invention, including a polynucleotide agent useful in performing a method of the invention, can be contacted directly with a target cell. For example, oligonucleotides useful as antisense molecules, ribozymes, or triplexing agents can be directly contacted with a target cell, whereupon they enter the cell and effect their function. A polynucleotide agent also can interact specifically with a polypeptide, for example, a receptor (or the cytokine itself), thereby altering the ability of a cytokine to interact specifically with the receptor. Such polynucleotides, as well as methods of making and identifying such polynucleotides, are disclosed herein or otherwise well known in the art (see, for example, O'Connell et al., Proc. Natl. Acad. Sci., USA 93:5883-5887, 1996; Tuerk and Gold, Science 249:505-510, 1990; Gold et al., Ann. Rev. Biochem. 64:763-797, 1995.).

[0062] An expression vector can contain the expression elements necessary to achieve, for example, sustained transcription of the encoding polynucleotide, or the regulatory elements can be operatively linked to the polynucleotide prior to its being cloned into the vector. An expression vector (or the polynucleotide) generally contains or encodes a promoter sequence, which can provide constitutive or, if desired, inducible or tissue specific or developmental stage specific expression of the encoding polynucleotide, a poly-A recognition sequence, and a ribosome recognition site or internal ribosome entry site, or other regulatory elements such as an enhancer, which can be tissue specific. The vector also can contain elements required for replication in a prokaryotic or eukaryotic host system or both, as desired. Such vectors, which include plasmid vectors and viral vectors such as bacteriophage, baculovirus, retrovirus, lentivirus, adenovirus, vaccinia virus, semliki forest virus and adeno-associated virus vectors, are well known and can be purchased from a commercial source (Promega, Madison WI; Stratagene, La Jolla CA; GIBCO/BRL, Gaithersburg MD) or can be constructed by one skilled in the art (see, for example, Meth. Enzymol., Vol. 185, Goeddel, ed. (Academic Press, Inc., 1990); Jolly, Canc. Gene Ther. 1:51-64, 1994; Flotte, J. Bioenerg. Biomemb. 25:37-42, 1993; Kirshenbaum et al., J. Clin. Invest. 92:381-387, 1993.).

[0063] Mechanisms of administration of cytokine/anticytokine therapy of the invention include, but are not limited to: i) direct administration of cytokines by mucosal, intravenous (iv), subcutaneous (sc), or intramusculatory (im) routes; ii) injection of DNA constructs encoding one or more cytokines; iii) conditioning *ex vivo* of relevant cell subtypes (e.g., T or B cells) by soluble cytokines in the same microenvironment; iv) administration of antibodies (e.g., human, non-human, and/or chimeric) designed to bind soluble or membrane bound cytokine receptors; v) administration of antibodies (e.g., human, non-human, and/or chimeric) designed to bind soluble or membrane bound cytokines; and vi) soluble cytokine receptors, engineered to interfere with the physiological targets of naturally released cytokines. Additional treatments are known to those of skill in the art.

[0064] Induction of epitope-specific therapy according to the present invention provides for initial identification of suitable epitopes, as described above, and subsequent generation, *in vivo* and/or *ex vivo*, of T cells capable of modulating immune responses. Examples of approaches for epitope-specific therapy include various approaches to induce modulation, *in vivo* and *ex vivo*, of epitope-specific responses including, but not limited to:

- i) tolerization to an immunomodulatory peptide via mucosal (i.e. nasal or oral) tolerization;
- ii) boosting of epitope-specific immune responses via sc, iv or im immunizations with proteins and epitopes or DNA encoding such proteins or epitopes;
- iii) regulation of epitope-specific responses by expansion *in vivo* and *ex vivo* of epitope-specific regulatory T cells; and
- iv) modulation *ex vivo* of epitope-specific T cells by means of artificial antigen presenting cells with or without conditioning environment. By “conditioning environment” is meant a microenvironment in which T cell responses may be modulated by adding to the medium soluble mediators (e.g., cytokines), and/or by soluble or support bound molecules (e.g., co-stimulatory molecules) capable of inducing a given T cell response.

[0065] In the invention methods, the induction period lasts for the time necessary for both treatments as described above to act, the induction period comprising the time for the cytokine/anticytokine oriented treatment to change the immune environment as desired via either up or down regulation, and for the epitope-specific T cells to be expanded. Generally, this period requires between one and three months, preferably two months in humans. This period may vary in accordance with the individual treatment.

[0066] Effective induction of epitope-specific T cells is preferably monitored by means of monitoring T cell modulation e.g. by a mechanism described in PCT/US99/24666 by T cell capture. Such monitoring may also be accomplished by other means known to those skilled in the art.

[0067] Following demonstration that regulatory T cells of a desired specificity of interest have been generated by the above described generation of epitope-specific responses (i.e., a T cell population with functional and phenotypical characteristics capable of modulating the immune response), the consolidation period is performed. Mechanisms for monitoring the generation and characteristics of T cell populations comprise, but are not limited to: i) T cell capture by means of fluorochrome tagged artificial antigen presenting cells; ii) Tetramer technology; iii) dimer technology; and iv) monitoring of CD69+ cells by flow cytometry. By "T cell capture" is meant identification, isolation and manipulation of antigen-specific cells by means of artificial antigen presenting cells, as described in PCT/US99/24666. By "tetramer technology" is meant a recombinant multiple (4x) of MHC/peptide complexes used to identify cells of a desired specificity. By "dimers technology" is meant a recombinant multiple (2x) of MHC/peptide complexes used to identify cells of a desired specificity (see for example, U.S. Patent No. 5,635,363.).

[0068] The epitope-specific peptides may be prepared in a variety of ways. Conveniently, they can be synthesized by conventional techniques employing automatic synthesizers, or may be synthesized manually. Alternatively, DNA sequences can be prepared which encode the particular peptide and may be cloned and expressed to provide the desired peptide. In this instance a methionine may be the first amino acid. In addition, peptides may be produced by recombinant methods as a fusion to proteins that are one of a specific binding pair, allowing purification of the fusion protein by means of affinity reagents, followed by proteolytic cleavage, usually at an engineered site to yield the desired peptide (see for example Driscoll et al. (1993) *J. Mol. Bio.* 232:342-350). The peptides may also be isolated from natural sources and purified by known techniques, including, for example, chromatography on ion exchange materials, separation by size, immunoaffinity chromatography and electrophoresis. Of particular interest in the present invention, peptides derived from hsp60 and dnaJ from any of human, mycobacteria and *E. coli* are useful.

[0069] During the consolidation period, the cytokine/anticytokine treatment is suspended or dramatically reduced, while the epitope-specific immunotherapy is

continued. Hence, the benefits of a non-toxic and well focused and controlled consolidated approach is provided, while the undesirable effects of the non-specific cytokine/anticytokine treatment is eliminated or reduced. As provided below in Example 1, this approach is shown to be effective as prolonged anticytokine therapy in inducing and maintaining a controllable immunotherapy-directed remission in autoimmune disease, while allowing complete withdrawal from the anticytokine treatment after the induction phase.

[0070] Application of this combination treatment regimen is valuable in numerous disease states that are sensitive to immune modulation. Up or down regulation of any number of cytokines and chemokines may be manipulated. For example not only can IL-10 and TGF $\beta$  be regulated in a controlled manner, as set forth in Example 1 below, but also other cytokines disclosed herein.

[0071] The two part treatment regimen provides identification of epitopes specific for a disease of interest rather than traditional methods that apply non-specific approaches. This identification of epitope is combined with specific immune modulation of a host's pathophysiology that is directly related to the particular disease. By such peptide-specific therapy, a change in the absolute numbers of peptide-specific T cells can be determined as well as quality of the T cell's response as they are involved in the pathology process. Example 1, set forth below, wherein the approach is applied using heat shock peptide 180-188, demonstrates that this method is applicable to peptide epitopes such as those derived from heat shock proteins. Specifically, a tailored/controlled immune modulation can be engineered using an immune therapy regimen that provides substantial benefit when combined with modulation of a host's pathophysiology. This novel controllable approach ultimately results in a significant reduction in the use of "traditional" therapeutic agents being administered to patients providing both an economic and clinical benefit.

[0072] One skilled in the art would readily appreciate that the present invention is well adapted for use in immunotherapy for modulation of pathogenic immune responses in immune mediated diseases.

[0073] The present invention provides a method of ameliorating a disorder such as an arthritis in a subject, wherein the epitope-specific immune responses have been identified. Such disorders may include, but are not limited to: rheumatoid arthritis and osteoarthritis.

[0074] As used herein, the term "ameliorate" means that signs or symptoms associated with an identified disorder are lessened. The signs or symptoms to be monitored will be characteristic of the particular disorder and will be well known to skilled clinician, as will the methods for monitoring the signs and conditions. For example, where the disorder is rheumatoid arthritis, the clinician will know that increased joint mobility or dexterity exhibited by the subject; or decreased inflammatory response as evidenced by lower cytokine levels or fewer inflammatory cells; or reduced level or decreased rate of joint and/or cartilage destruction as evidenced by an imaging method, are indicative of a method of the invention ameliorating the arthritis. Similarly, where the disorder is osteoarthritis, the clinician will know that a decrease in joint and/or cartilage damage is indicative of ameliorating the arthritis. focused and controlled consolidated approach is provided, while the undesirable effects of the non-specific cytokine/anticytokine treatment is eliminated or reduced. As provided below in the examples, this approach is shown to be effective as prolonged anticytokine therapy in inducing and maintaining a controllable immunotherapy-directed remission in autoimmune disease, while allowing complete withdrawal from the anticytokine treatment after the induction phase.

[0075] Detection of T cell specific epitopes is of interest in connection with a variety of conditions associated with T cell activation. Such conditions include autoimmune diseases, e.g. multiple sclerosis, myasthenia gravis, rheumatoid arthritis, type 1 diabetes, graft vs. host disease, Grave's disease, etc.; various forms of cancer, e.g. carcinomas, melanomas, sarcomas, lymphomas and leukemias. Various infectious diseases such as those caused by viruses, e.g. HIV-1, hepatitis, herpes viruses, enteric viruses, respiratory viruses, rhabdovirus, rubeola, poxvirus, paramyxovirus, morbillivirus, etc. are of interest. Infectious agents of interest also include bacteria, such as *Pneumococcus*, *Staphylococcus*, *Bacillus*, *Streptococcus*, *Meningococcus*, *Gonococcus*, *Escherichia*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*,

Hemophilus, Yersinia, Listeria, Corynebacterium, Vibrio, Clostridia, Chlamydia, Mycobacterium, Helicobacter and Treponema; protozoan pathogens, and the like. T cell associated allergic responses may also be monitored, e.g. delayed type hypersensitivity or contact hypersensitivity involving T cells.

[0076] Of particular interest are conditions having an association with a specific peptide or MHC haplotype, where the subject binding complexes may be used to track the T cell response with respect to the haplotype and antigen. A large number of associations have been made in disease states that suggest that specific MHC haplotypes, or specific protein antigens are responsible for disease states. However, direct detection of reactive T cells in patient samples has not been possible. Detection and quantitation with the subject binding complexes allows such direct detection. As examples, the activity of cytolytic T cells against HIV infected CD4+ T cells may be determined with the subject methods. The association of diabetes with the DQB1\*0302 (DQ3.2) allele may be investigated by the detection and quantitation of T cells that recognize this MHC protein in combination with various peptides of interest. The presence of T cells specific for peptides of myelin basic protein in conjunction with MHC proteins of multiple sclerosis patients may be determined. The antigenic specificity may be determined for the large number of activated T cells that are found in the synovial fluid of rheumatoid arthritis patients. It will be appreciated that the subject methods are applicable to a number of diseases and immune-associated conditions.

[0077] The isolation of antigen specific T cells finds a wide variety of applications. The isolated T cells may find use in the treatment of cancer as in the case of tumor-infiltrating lymphocytes. Specific T cells may be isolated from a patient, expanded in culture by cytokines, antigen stimulation, etc., and replaced in the autologous host, so as to provide increased immunity against the target antigen. A patient sample may be depleted of cells reactive with a specific antigen, to lessen an autoimmune response.

[0078] Inhibition of immune function may be achieved by inducing anergy of specific T cells, or by ablation of reactive T cells. The subject binding complexes allow a therapy to be targeted to very specific subsets of T cells. The ability to inhibit immune system functions is known to be therapeutically useful in treating a variety of diseases

such as atherosclerosis, allergies, autoimmune diseases, certain malignancies, arthritis, inflammatory bowel diseases, transplant rejection and reperfusion injury. Specific diseases of interest include systemic lupus erythematosus; rheumatoid arthritis; polyarteritis nodosa; polymyositis and dermatomyositis; progressive systemic sclerosis (diffuse scleroderma); glomerulonephritis; myasthenia gravis; Sjogren's syndrome; Hashimoto's disease; Graves' disease; adrenalitis; hypoparathyroidism; pernicious anemia; diabetes; multiple sclerosis, and related demyelinating diseases; uveitis; pemphigus and pemphigoid cirrhosis; ulcerative colitis; myocarditis; regional enteritis; adult respiratory distress syndrome; local manifestations of drug reactions, such as dermatitis, etc.; inflammation-associated or allergic reaction patterns of the skin; atopic dermatitis and infantile eczema; contact dermatitis; psoriasis; lichen planus; allergic enteropathies; allergic rhinitis; bronchial asthma; transplant rejection, e.g. heart, kidney, lung, liver, pancreatic islet cell, etc.; hypersensitivity or destructive responses to infectious agents; poststreptococcal diseases, e.g. cardiac manifestations of rheumatic fever, and the like.

[0079] In addition to the combination immunotherapy described here, it may be desirable to also ablate specific T cells, the subject binding complexes may be conjugated to a toxin moiety. Various cytotoxic agents are known and have been used in conjunction with specific binding molecules. Illustrative of these compounds are ricin, abrin, diphtheria toxin, maytansinoids, cisplatin, and the like. Where there are two subunits, only the cytotoxic subunit may be used, e.g. the .alpha.-unit of ricin. The toxin is conjugated to the binding complex, generally by means of a cross-linker, or other conjugation that includes a disulfide bond. Toxin conjugates are disclosed in U.S. Pat. Nos. 5,208,020; 4,863,726; 4,916,213; and 5,165,923. The toxin conjugate is administered so as to specifically eliminate the target T cells without exerting significant toxicity against other cells.

[0080] The subject binding complexes may be administered to a host to induce anergy of the specific T cells. The binding complex will induce T cell anergy upon binding, because the co-stimulator molecules necessary for T cell activation are not present. The binding complexes are administered to individuals, preferably mammals, in

a manner that will maximize the likelihood of the binding complexes reaching the targeted T cell and binding to it, and thereby inducing anergy. This in turn will inhibit the immune response mediated by that T cell.

[0081] The following examples are intended to illustrate but not limit the invention.

#### EXAMPLE 1

[0082] In this example, a comparison is made between currently used traditional treatment methods and the invention methods. Performance of traditional methods of treating humans to lower the inflammatory processes arising due to TNF $\alpha$  includes administration of etanercept, (commercially known as Enbrel $\mathcal{E}$  (Immunex, Seattle, WA) a soluble recombinant TNF $\alpha$  receptor/IgG1 molecule that is administered to reduce the presence of TNF $\alpha$ . This example provides data using a rat model (which is recognized as directly correlating with human response) showing that the combination therapy method of the invention provides substantial reduction of side effects with clinically significant benefits and reduction of the need to administer etanercept. (Typically, the use of etanercept in patients is not only very expensive, but also has unforeseen long term side effects, presents an increased risk for opportunistic infection and malignancy, and relapse of disease will occur if administration is stopped.)

[0083] In the animal model of this Example, such immunomodulatory treatment was used in combination with induction of mucosal tolerance. The clinical outcome was then monitored and immunological characterization of T cells was performed. Adjuvant Arthritis was induced by injection into the base of the subject rat tail of 0.1 mg of etanercept (Day 0). At Day 8, daily arthritis scores (having a maximum reading of 16) were measured and the rats were weighed every other day. Treatment was begun at Day 9.

[0084] Subject rats were divided into four treatment groups: etanercept 3X sc at day 9, 11, 13; etanercept 1X sc at day 9; etanercept 1X sc at Day 9 and heat shock

peptide 180-188 administered on days 10, 13, 16, and 19; and no treatment group. Etanercept was dosed at 0.3 mg/kg of body weight. Heat shock peptide 180-188 was dosed at 100 ug. (The heat shock peptide was that of mycobacterial protein 180-188 and known to be the main epitope recognized by T cells that are pathogenic in adjuvant arthritis).

**[0085]** Results of the above stated administration are shown in Figure 1. As is clear from the graph, combined treatment regimen comprising one injection of etanercept with multiple exposure of heat shock peptide to mucosa reduced the arthritis score. Additionally, mandibular and inguinal lymph nodes were harvested from the subject rats at days 22-35 and the cells were cultured *in vitro*, in order to confirm the theory that the underlying mechanism for the positive efficacy of the combination treatment regimen includes a functional switch from a Th1 to a Th2 response. Proliferation of the cells was carried out by [<sup>3</sup>H] TdR incorporation. Intracellular cytokine production and surface marker expression was tested using FACS. Cytokine and Tbet and GATA-3 gene transcription were carried out by Taqman.

**[0086]** In Figure 2 the results of the above regimen show that as of days 22 and 35, MLN are stimulated substantially as shown in the 35 day enbrel/180-188 bar graph. Additional data are provided in Figures 3-7. In Figure 3, intracellular production of (ILN) is shown using FACS analysis. As indicated, the combination therapy sample cells show a substantial stimulation of CD4+ cells as shown by expression of IL-10 or CTLA-4. Likewise, in Figure 4, gene transcription of IL-10 is markedly increased in the combination treatment sample over the Enbrel alone and no treatment samples. The same is true for gene transcription of TGF $\beta$ , and GATA-3 as shown in Figures 5 and 6, respectively. However, there is a marked decrease in Tbet expression as shown in Figure 7. Each of these genes are important in proving efficacy of the combination regimen because Tbet and GATA-3 are gatekeeper genes, which control development of a Th-1 or Th-2 cytokine cascade.

**[0087]** The results show that there is an emergence of antigen-specific proliferation of cells specific for the heat shock peptide 180-188 by day 35, increased gene transcription of IL-10 and TGF $\beta$  in MLN and ILN, an increased intracellular

production of IL-10, and expression of CTLA-4 in ILN. Therefore, a functional switch towards Th2 by increase in GATA-3, and decrease of Tbet. The overall result is that combination treatment of antigen-specific immunotherapy and anti-TNF $\alpha$  treatment leads to significant reduction of arthritis in Adjuvant Arthritis. Additionally, as one of skill in the art would recognize given the clinical applicability of the above mouse model, there is a correlation between clinical efficacy and generation of regulatory T cells as shown by the proliferation of antigen, IL-10 and TGF $\beta$  production and Th2 phenotype stimulation.

[0088] Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

## WHAT IS CLAIMED IS:

1. A method of modulating an immune response in a subject having an immune response-related disorder comprising:
  - a) administering a therapeutically effective amount of a an antigen-specific epitope, wherein administration provides epitope-specific T cell immune modulation; and
  - b) administering a therapeutically effective amount of a cytokine or a regulatory effective amount of an agent that affects cytokine activity or expression, wherein administration of a) and b) modulates the immune response in the subject.
2. The method of claim 1, wherein the administration is performed by direct administration of a cytokine by mucosal, intravenous, subcutaneous, or intramuscular administration.
3. The method of claim 1, wherein the administration of a cytokine comprises administration of a nucleic acid encoding the cytokine.
4. The method of claim 1, wherein administration of an agent that affects cytokine activity comprises administration of an antibody.
5. The method of claim 4, wherein the antibody binds a soluble or membrane bound cytokine receptor.
6. The method of claim 5, wherein the cytokine receptor interferes with the target of a naturally released cytokine.
7. The method of claim 4, wherein the antibody binds a soluble or membrane bound cytokine.
8. The method of claim 4, wherein the antibody is a human antibody, a non-human antibody, or a chimeric antibody.
9. The method of claim 1 wherein the administration in a) and b) occurs simultaneously.

10. The method of claim 1 wherein the administration in a) and b) occurs sequentially.
11. The method of claim 10, wherein the administration is in varying ratios.
12. The method of claim 1, wherein the cytokine is associated with an inflammatory or a tolerating immune response.
13. The method of claim 12, wherein the inflammatory or tolerating immune response is modulated by an immunomodulatory cytokine.
14. The method of claim 13, wherein the immunomodulatory cytokine is TNF $\alpha$ , TNF $\beta$ , IFN $\alpha$ , IFN $\beta$  or IFN $\gamma$ , IL-1, IL-4, IL-6, IL-10, IL-15 or IL-23.
15. The method of claim 1, wherein the antigen-specific epitope is derived from a heat shock protein.
16. The method of claim 1, wherein the disorder is selected from the group consisting of arthritis, diabetes type I, multiple sclerosis, inflammatory bowel disease, systemic lupus erythematosus, cancer; polyarteritis nodosa; polymyositis and dermatomyositis progressive systemic sclerosis (diffuse scleroderma); glomerulonephritis; myasthenia gravis; Sjogren's syndrome; Hashimoto's disease; Graves' disease; adrenalitis; hypoparathyroidism; pernicious anemia; diabetes; multiple sclerosis, and related demyelinating diseases; uveitis; pemphigus and pemphigoid cirrhosis; ulcerative colitis; myocarditis; regional enteritis; adult respiratory distress syndrome; local manifestations of drug reactions, such as dermatitis; inflammation-associated or allergic reaction patterns of the skin; atopic dermatitis and infantile eczema; contact dermatitis; psoriasis; lichen planus; allergic enteropathies; allergic rhinitis; bronchial asthma; transplant rejection; hypersensitivity or destructive responses to infectious agents; and poststreptococcal diseases.
17. The method of claim 1, wherein administration of an agent that affects cytokine activity comprises administration of a peptide or peptidomimetic.
18. The method of claim 17, wherein the agent binds to a cytokine receptor.

19. The method of claim 17, wherein the agent binds to a cytokine.
20. The method of claim 1, further comprising *ex vivo* conditioning of T cells or B cells with soluble cytokines.
21. The method of claim 20, wherein the *ex vivo* conditioning is performed in the same microenvironment.
22. The method of claim 1, wherein administration of an agent that affects cytokine activity comprises administration of an agent comprising a cytokine receptor.
23. The method of claim 22, wherein the cytokine receptor interferes with a natural target of a naturally released cytokine.
24. The method of claim 1, wherein the antigen-specific epitope is selected from a peptide of Table I.
25. A method of modulating immune processes and pathogenesis of immune mediated diseases comprising:
  - a) identifying at least one suitable epitope;
  - b) identifying at least one cell type associated with the immune mediated disease; and
  - c) administering a combination of antigen-specific and cytokine or anticytokine therapywherein the administering induces an immune response to the immune mediated disease.
26. The method of claim 25, wherein the immune mediated disease is an autoimmune disease.
27. The method of claim 26, wherein the autoimmune disease is arthritis.
28. The method of claim 26, wherein the autoimmune disease is immunodeficiency or cancer.

29. The method of claim 25, wherein the anticytokine therapy comprises anti TNF $\alpha$  therapy.

30. The method of claim 25, wherein the cytokine is associated with an inflammatory or a tolerating immune response.

31. The method of claim 30, wherein the inflammatory or tolerating immune response is modulated by an immunomodulatory cytokine.

32. The method of claim 31, wherein the immunomodulatory cytokine is TNF $\alpha$ , TNF $\beta$ , IFN $\alpha$ , IFN $\beta$  or IFN $\gamma$ , IL-1, IL-4, IL-6, IL-10, IL-15 or IL-23.

33. The method of claim 25, wherein the antigen-specific epitope is derived from a heat shock protein.

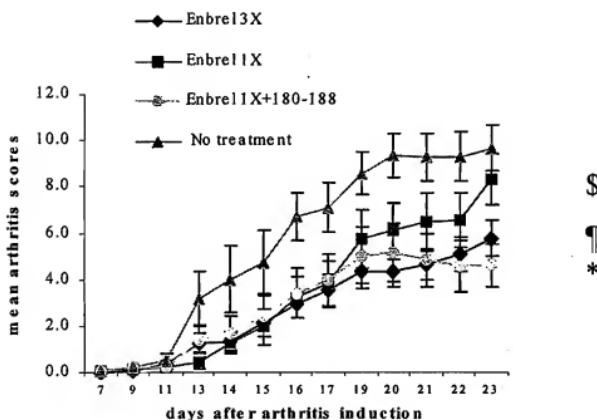
34. The method of claim 25, wherein the antigen-specific epitope is selected from a peptide of Table 1.

35. A method of treating a subject having an immune response-related disorder comprising:

- a) administering a therapeutically effective amount of an antigen-specific epitope, wherein administration provides epitope-specific T cell immune modulation; and
- b) administering a therapeutically effective amount of a cytokine or a regulatory effective amount of an agent that affects cytokine activity or expression, wherein administration of a) and b) modulates the immune response in the subject, thereby treating the subject.

36. The method of claim 35 wherein the antigen-specific epitope is selected from a peptide of Table 1.

## Modulation of AA after combination treatment with Enbrel and mycobacterial hsp60 peptide 180-188



\$ p:0.364 vs no treatment

¶ p:0.0054 vs no treatment

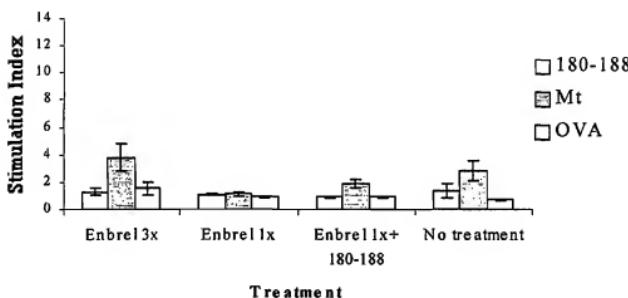
\* p:0.0013 vs no treatment

**FIGURE 1**

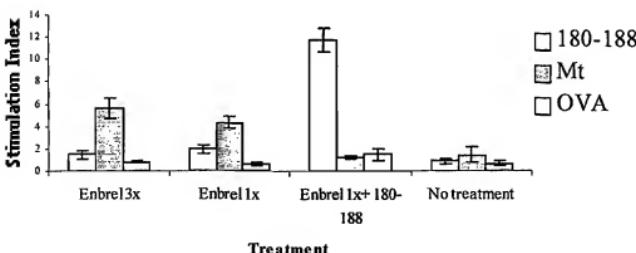
2/7

## Proliferation after *in vitro* stimulation of mandibular lymphnode cells (MLN)

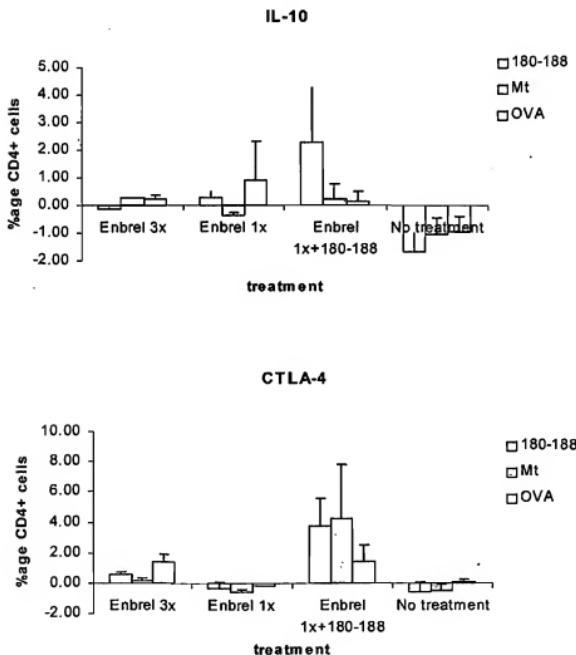
day 22



day 35

**FIGURE 2**

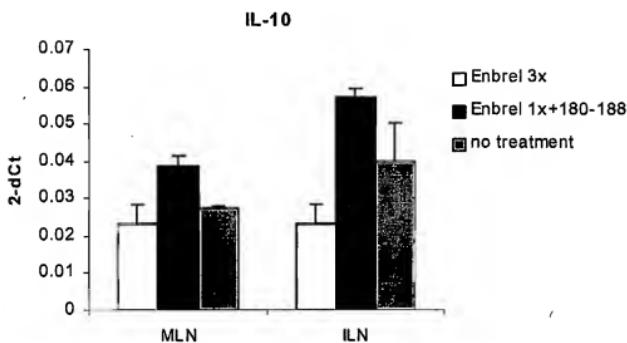
## Intracellular Cytokine production in ILN by FACS



**FIGURE 3**

4/7

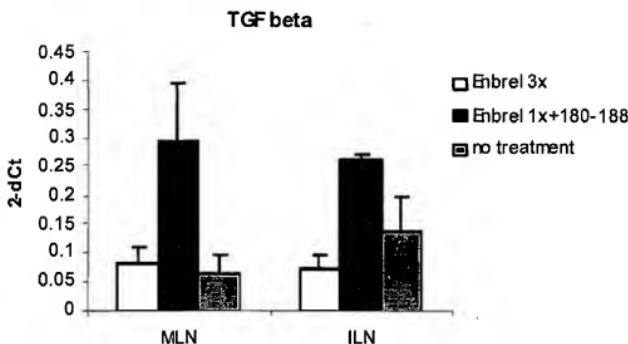
## Gene transcription of IL-10 by Taqman



**FIGURE 4**

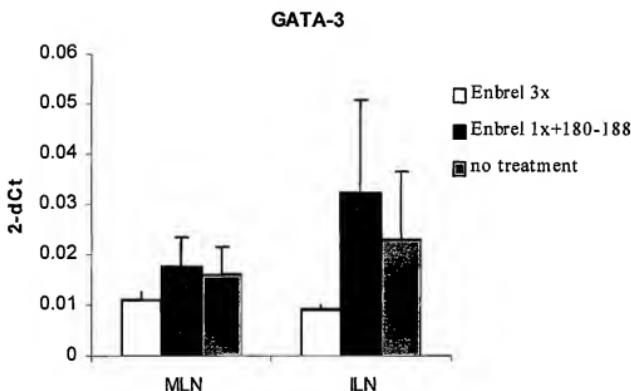
5/7

## Gene transcription TGF- $\beta$ by Taqman



**FIGURE 5**

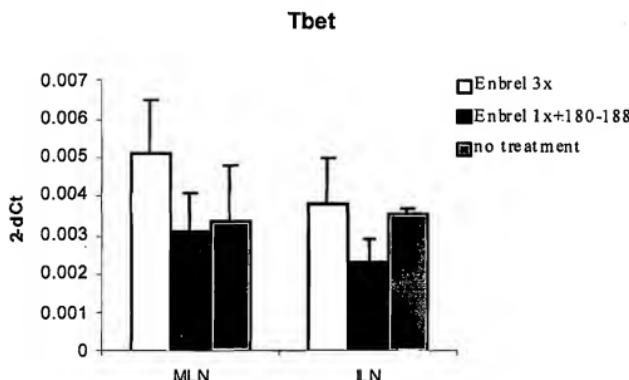
## Gene transcription of GATA-3 by Taqman



**FIGURE 6**

7/7

## Gene transcription of Tbet by Taqman



**FIGURE 7**

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(22) International Filing Date: 25 September 2002 (25.09.2002) (73) Inventors/Applicants (for US only): ALBANI, Salvatore [IT/US]; 629 4th Street, Encinitas, CA 92024 (US). MARTINI, Alberto [IT/IT]; Via Taverna, 25, I-Piacenza (IT).

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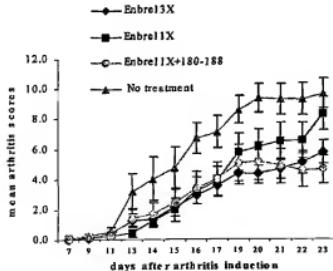
60/325,499 25 September 2001 (25.09.2001) US  
60/339,284 11 December 2001 (11.12.2001) US

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Filed on 11 December 2001 (11.12.2001)

[Continued on next page]

(54) Title: COMBINATION THERAPIES FOR IMMUNE MEDIATED DISEASES

### Modulation of AA after combination treatment with Enbrel and mycobacterial hsp60 peptide 180-188



(57) Abstract: The current invention provides for methods of immunotherapy using a combination of epitope-specific and cytokine or anticytokine immunotherapy. The method provides for modulation of pathogenic immune response and includes the identification of molecules comprising specific epitopes involved in a particular disease state of interest, administration of the epitope-specific molecule in conjunction with the cytokine or anticytokine, and downstream modification of the administration of the cytokine/anticytokine relative to the administration of the epitope-specific molecule. Figure 1 is a graph showing the effects on mean arthritis scores calculated into four groups following induction of Adjuvant Arthritis.

WO 03/026579 A3

§ p:0.364 vs no treatment  
¶ p:0.0054 vs no treatment  
\* p:0.0013 vs no treatment



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## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/00, 38/16, 39/00, 39/38, 39/35  
 US CL : 514/2, 8, 12, 13, 14; 424/185.1, 184.1, 248.1, 130.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 U.S. : 514/2, 8, 12, 13, 14; 424/185.1, 184.1, 248.1, 130.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,037,135 A (KUBO et al) 14, MARCH 2000, columns 5-19, the claims.	1-35
Y	WO 00/23053 A2 (ALBANI) 27 APRIL 2000, pages 19-33, 40-46, the claims	1-35
Y	US 6,077,519 A (STORKUS et al) 20 JUNE 2000, column 4-6, the claims.	1-35
Y	US 6,218,37 B (KRIEG et al) 17 APRIL 2001, the claims.	1-35
Y	US 2001/0007758 A1, (WEINER et al.) 12 July 2001, page 1-2, and the claims.	1-35

Further documents are listed in the continuation of Box C.  See patent family annex.

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"R"	document member of the same patent family

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**INTERNATIONAL SEARCH REPORT**

PCT/US02/30578

**Continuation of Item 4 of the first sheet:**

The title is too long.

Combination therapies for Immune Mediated Diseases

**Continuation of B. FIELDS SEARCHED Item 3:**

MEDLINE CAPLUS

search terms: epitope, antigen, T cell, immune modulation, cytokine, antibody, peptide, IFN, TNF, IL, arthritis, cancer, inflammatory bowel